Yeast Genomic Expression Studies Using DNA Microarrays

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Introduction

The technological developments in the area of genome science have revolutionized the methods of biological exploration, allowing organisms to be studied on a genomic scale. Characterizing whole-genome expression using DNA microarrays provides a snapshot of an organism's genome in action by revealing the relative transcript levels of thousands of genes at a time. Exploration of the dynamic nature of genomic expression programs throughout the natural life cycle of cells presents a variety of biological insights, hinting at the underlying cellular processes that mediate or respond to changes in gene expression.

In a seminal study by DeRisi *et al.*, DNA microarrays were used to follow the global changes in gene expression during the natural transition of yeast cells from fermentative metabolism to respiration, known as the diauxic shift[1]. Despite decades of study on the metabolic pathways involved, the work demonstrated the potential of genomic expression profiling by revealing many previously unrecognized features of the metabolic transition. Nearly 30% of the yeast genome (approximately 1,700 genes) was significantly altered in expression as the available glucose in the medium was depleted, and subsets of genes displayed distinct temporal patterns of transcript variation. DeRisi *et al.* demonstrated that genes displaying similar expression patterns were functionally related and regulated by upstream sequence motifs common to the genes' promoters. This result established the enormous potential of genomic expression characterization in implicating hypothetical functions for uncharacterized genes, because of their similarity in expression with genes of known functions, while suggesting modes of transcriptional regulation by pointing to signaling factors known to act through the conserved promoter elements common to coregulated genes.

Since then, genomic expression studies have revealed a wealth of information about yeast biology. Characterization of transcript fluctuation during the yeast cell cycle not only identified approximately 700 cell-cycle regulated genes, but also painted a more complete picture of the biological processes that occur during each cell-cycle phase [2,3]. In addition, the identification of groups of coregulated cell-cycle genes led to the subsequent identification of the signaling factors that regulate their expression and the promoter elements through which they execute their control[3,4,5]. Investigation of the temporal changes in gene expression during sporulation of diploid cells helped to more accurately define the temporal phases of the developmental process, while identifying thousands of genes, many of them completely uncharacterized, that were involved in meiosis[6]. A separate study characterizing genomic expression patterns dependent on ploidy further clarified cellular processes and gene expression patterns that were affected by genomic copy number[7].

Microarray studies have presented a complex picture of developmental processes in yeast, and they have also revealed the intricacies of cellular responses to environmental changes. Comparison of the genomic expression programs triggered by diverse environmental stresses revealed many condition-specific features in the gene expression responses[8], but at the same time indicated that the majority of the genomic expression responses to each unique environment was in fact part of a general environmental stress response, common to all conditions[8,9]. Subtleties in the expression profiles, however, revealed that this general stress response was regulated by different upstream signaling factors in response to different experimental conditions, demonstrating the complexities in the regulation of yeast genomic expression [8,10].

Exploration of global gene expression in wild-type cells has revealed the cells' natural expression programs during development and environmental responses, however aberrant expression in mutant strains has been equally informative. For example, Hughes *et al.* generated a large compendium of single-gene deletion mutants and demonstrated that the genomic expression programs resulting from many of the individual gene deletions was indicative of the deleted gene's cellular role, as cells lacking functionally related genes displayed similar anomalies in genomic expression relative to wild-type cells[11]. Many other studies have identified abnormalities in gene expression in strains harboring mutations in signaling factors such as protein kinases and transcription factors, revealing targets of those signaling molecules while implicating specific conditions under which those signaling pathways are active[12,13,14,15,11,16,10,1,17,8,18,19,20,21].

The use of microarrays to characterize genomic expression programs is expanding to study the evolution of yeasts by exploring variation of genomic expression responses in natural *S. cerevisiae* isolates[22] and strains evolved under experimental conditions[23]. As more complete genome sequences emerge, including that of *Candida albicans*[24], *Cryptococcus neoformans*[25], the fission yeast *Schizosaccharomyces pombe*[26], and other yeast species[27], comparison of genomic expression programs within and across different yeasts will illuminate the physiological processes within each organism, and reveal evolutionary differences in the regulation and orchestration of genomic expression programs.

The advent of DNA microarrays has allowed the exploration of gene expression on a whole-genome scale. DNA microarrays consist of DNA fragments that have been immobilized onto a glass support[28]. Unlike some other commercially available arrays, in which oligonucleotides are synthesized on the solid surface[29,30], microarrays are constructed by spotting DNA fragments onto a pretreated microscope slide. Hybridization of a fluorescently labeled nucleic acid solution to the microarray allows the rapid identification and quantitation of all molecules in the solution, by exciting and detecting the fluorescent molecules bound to their immobilized DNA complement. In actuality, accurate measurement of absolute nucleic acid levels is confounded by a number of experimental variables. To avoid these limitations, microarrays utilize competitive hybridization of two, differentially labeled probe samples. By measuring the relative abundance of molecules in the two samples, the effects of subtle variation in the hybridization conditions are minimized, resulting in highly reproducible measurements. In addition to providing reliable measurements, microarrays are relatively affordable and straightforward to use. They can be applied to any technique in which identification by nucleic acid hybridization can be utilized, including measuring relative transcript abundance to characterize genomic expression patterns.

This chapter will focus on the use of microarrays to characterize genomic expression programs in yeast, although many of the details can be applied to microarray uses beyond gene expression studies. Because previous sources have extensively described the construction and use of microarrays[31,32,33], this review will focus on details that are specific to yeast microarray experiments, as well as critical features of microarray use. Readers are encouraged to consult the listed resources that complement this review.

Microarray construction

The construction of microarrays involves four general steps: generation of the target DNA to be spotted, preparation of glass microscope slides, printing of the arrays, and processing the final product before use. Microarray spotting machines can be purchased commercially, however construction of the microarrayer developed in Pat Brown's lab at Stanford[28] is relatively straightforward, thanks to detailed instructions provided by Joe DeRisi and members of the Brown lab[32,33]. The information contained on these websites provides specific instructions and tips for the printing process, which will not be covered here.

Generation of target DNA for spotting. Any DNA solution can be spotted onto microarrays, however the most common yeast microarrays to date consist of PCR products representing every predicted open reading frame in the *S. cerevisiae* genome. Genomic sequences can be amplified using standard PCR conditions and sequence-specific primers designed to amplify *S. cerevisiae* open reading frames as well as intergenic sequences (Research Genetics # 40603 and #40903 at a current price of $38,000 per primer set). For other genomes for which the complete sequence is not yet known, genomic or cDNA libraries can be amplified using common, plasmid-specific primers, as has been done for other organisms[34]. Standard 100 ?l PCR reactions are performed in 96-well plates using 1 ?M primer, 40 ng yeast genomic DNA from the S288C sequenced strain (Research Genetics #40802), 2 mM Magnesium chloride, and Taq polymerase conditions. The amplification begins with a 10 minute denaturation at 92^{o}C, followed by 35 cycles of 92^{o}C for 30 seconds, 56^{o}C for 45 seconds, and 72^{o}C for 3.5 minutes. After verification of the reaction products by gel electrophoresis and reamplification of failed reactions, the DNA is precipitated as previously described [31,32,33]. The PCR products are resuspended overnight in 50 ?l of 3X SSC (or alternatively a larger volume of double-distilled, deionized water (ddH$_2$O) to facilitate robotic transfer) and then distributed into non-sterile, v-bottom 384-well plates (Genetix #6004) in 4 ?l per well aliquots to yield twelve replicate plate sets. (Be aware that other brands of 384-well plates or plates that have been sterilized by irradiation are prone to warping and may display variable well spacing, causing problems during the printing process.) After lyophilization in a speed vacuum, the dried DNA pellets can be stored at room temperature for months. If the original PCR products were resuspended in water, the plates should be resuspended one time in 4 ?l 3X SSC before use. Subsequently, the dried DNA pellets should be resuspended overnight in 4 ?l ddH$_2$O before each use, and after use the plates can be dehydrated, stored, and reused for at least 5 printings. (After ~4 printings from the same plate set, the DNA should be resuspended one time in 4 ?l 1.5X SSC to replace the salt lost during previous printings). Each print run yields 140 – 250 microarrays, depending on the microarrayer used, and reuse of the print plates allows the production of at least 750 microarrays per plate set. Thus, each genome amplification yields approximately 10,000 microarrays.

Recently, 70mer oligonucleotides representing each of the predicted open reading frames in *S. cerevisiae* have been designed (Operon Technologies, current price $35,000 for enough sample to generate ~5,000 microarrays), providing an alternative source of target DNA to be spotted onto the microarrays. The sequence chosen for each oligo is designed to minimize sequence similarity between the set of 70mers, thereby reducing cross hybridization of the spots on the microarrays. In addition, because the concentration of all the 70mers in the printing set is roughly equal, the DNA mass in the microarray spots is normalized, unlike spotted PCR products. Although the cost per microarray is slightly higher when printing oligos (~$7 per microarray) versus PCR products (<$1 per microarray), the initial monetary investment is substantially less (since much of the cost of PCR amplification is accounted for by PCR reagents), and the labor required to prepare the genomic sequences is drastically reduced. These features make the spotting of 70mers an attractive alternative to PCR amplification of the yeast genome.

Preparing slides before printing. A critical step in making good microarrays is generating high-quality microscope slides for the arrays. Although various types of slides are commercially available, generation of poly-lysine-coated slides is simple and reliable. Glass microscope slides (Gold Seal #3010) can be loaded into metal slide racks and processed in 400 ml glass slide chambers (Shandon Lipshaw #121). The first step is to thoroughly clean the slide surface before coating. For each rack of thirty slides, dissolve 35 g Sodium hydroxide in 140 ml of ddH_2O, then add 210 ml 95% Ethanol and mix thoroughly. (Never use 100% Ethanol in the array making process, as benzene contaminants can carry through the process and fluoresce on the microarrays.) Place the slide rack containing the slides into the solution and incubate for 2 hours with slight agitation on a platform shaker. The slides should then be rinsed 5-7 times in ~400 ml ddH_2O per slide rack, a step that appears to be critical for proper slide generation. Exposure of the slides to air should be minimized during this process. Next, prepare the poly-lysine solution by mixing 35 ml poly-L-lysine (Sigma #P 8920), 35 ml filtered PBS buffer, and 280 ml ddH_2O per slide rack in a plastic cylinder. Place the slide rack into the solution and incubate for 30 minutes at room temperature with slight agitation on a platform shaker. Rinse the slides one time by plunging ~5 times in 350 ml ddH_2O per rack, then dry the slides by centrifugation at 500 rpm for 5 minutes in a table-top centrifuge. Before use, the slides must be aged for ~14 days, to achieve optimal hydrophobicity of the slide surface, in a plastic slide box (slide boxes with rubber surfaces should not be used as emissions from the rubber can deteriorate the poly-lysine surface). The aging process can be accelerated by baking the slides for 5-10 minutes at $50^{o}C$ in an oven after centrifuge drying. Before use, the slides should be tested by printing a small number of DNA spots onto representative slides from each batch, hybridizing fluorescently labeled probe to the microarrays, then scanning the finished arrays to verify adequate hybridization signal in the spots and low background signal elsewhere on the arrays. It is feasible to generate 4-6 racks of slides at a time, and it is advisable to prepare multiple batches of slides before microarray printing to ensure a sufficient number of high-quality slides.

Processing microarrays after printing. Another critical step in the construction of microarrays is post-processing of the microarrays before hybridization. After the DNA is spotted onto the microarrays, the unbound lysine groups on the slide surface must be chemically blocked to prevent nonspecific binding of probe DNA to the array.

Because post-processing removes the salt deposited during printing, and thus prevents visualization of the DNA spots, the first step of the process is to mark the boundary of the spots on each microarray. Place the microarray DNA-face down over two spaced supports, such that the sides of the microarray are supported and the DNA spots are free from any surface. Etch the boundary of the microarray spots onto the back of the slide with a glass etcher. The etched boundary allows accurate placement of the fluorescent probe and the coverslip for microarray hybridization.

The microarrays must then be rehydrated to evenly distribute the mass of DNA within each spot. Place the array DNA-face down in a slide hydrating chamber (Sigma #H 6644) over a room-temperature solution of 1X SSC, and incubate until the spots on the array are visibly hydrated and glistening (5-15min). Overhydration of the microarrays can cause the spots to bleed together, and therefore the process should be monitored and terminated if the spots are in danger of contaminating others. The arrays are then rapidly dried by placing the array, DNA-face up, onto a preheated metal block at 80°C for 3-5 seconds, or until the vapor has evaporated from the slide. The DNA can be crosslinked to the slide in a uv crosslinker at 60 mJoules by laying the individual microarrays DNA-face-up in the crosslinker.

Before performing the chemical step, gather the reagents, including 1 liter of preheated ddH_2O in a beaker large enough to hold a slide rack, one empty glass slide chamber, one glass slide chamber filled with 95% Ethanol, and chemical reagents. Place the slides into a metal rack and set aside. The critical chemical blocking step must be initiated rapidly and carried out exactly as described here: in a separate flask, add 6 g Succinic anhydride (Aldrich #239690) to 325 ml 1-Methyl-2-pyrrolidinone (Aldrich #328634) and stir gently with a stir bar (Succinic anhydride is stable long-term if stored under vacuum; 1-Methyl-2-pyrrolidinone should be clear before mixing, and yellowed solvent should not be used). Immediately after the last grains of Succinic anhydride dissolve, add 25 ml 1 M Sodium borate pH 8.0 (made by dissolving boric acid in ddH_2O and adjusting the pH with Sodium hydroxide). Immediately pour the mixed solution into the *empty* glass chamber, then swiftly plunge the rack of microarrays *into* the solution. After a 15 minute incubation with gentle agitation on a platform shaker, quickly remove the rack of arrays from the blocking solution and plunge into preheated ddH_2O for cleansing and denaturation: for microarrays containing PCR products, plunge the slide rack into just-below boiling water for 2 minutes, and for oligonucleotide-containing microarrays, plunge into 55°C water for only 5 seconds to minimize loss of DNA from the microarray slide. Finally, plunge the slide rack into 95% Ethanol about 5 times and dry the slides by centrifugation at 500 rpm for 3 minutes. The arrays are ready for hybridization and can be stored at room temperature for at least 3-6 months.

***Planning the experiment*.**

The key to a successful genomic expression experiment is proper planning and preparation. This section describes important considerations in the setup of genomic expression studies, and includes examples of common oversights in experimental design and execution.

Strains and growth conditions. Although many labs often use their favorite strains, careful consideration should be given to the strain background used for microarray experiments. Different lab strains can have significantly different genotypes, leading to strain-dependent differences in genomic expression responses. When comparing the results of genomic expression experiments, particularly when comparing wild-type and mutant responses, an isogenic strain background must be used. Strains that have unnecessary auxotrophic markers should be avoided, as these additional phenotypes can complicate cellular responses and confound the analysis of genomic expression patterns.

Genomic expression can also be affected by growth conditions, including culture system (shaker flasks *versus* chemostat), culture aeration, growth temperature, and medium. Synthetic medium offers an advantage over rich yeast-extract medium, in that nutrient concentrations can be precisely controlled. The choice of synthetic *versus* rich medium can significantly affect genomic expression responses to environmental stimuli[35]. The experimenter should be aware that these features may affect genomic expression programs and that variations in the growth conditions may result in somewhat different gene expression patterns.

Experimental design. Perhaps the most important aspect of setting up genomic expression studies is choosing experimental conditions for which there are as few variables as possible. Often, experimental variables are overlooked, such as extensive cell handling during the experiment, the effects of a drug carrier solution on the cells, pleiotropic effects of the chosen stimulus, and progression of the cells into the diauxic shift during the experiment (see below). These features are to be avoided as they confound data analysis and can lead to poor experimental reproducibility. If these features cannot be avoided (for example, extensive culture handling), a separate, control experiment must be performed to identify the genomic expression responses triggered by these unavoidable variables.

Choosing the appropriate dose of a stimulus and assessing the timing of the cellular response often requires prior investigation. The desired dose can be assessed by performing dosage curves and monitoring the response of cellular indicators, such as changes in the expression of representative genes, cell morphology, growth rate, cell-cycle arrest, cell viability, etc. Conditions that result in high cell death should be avoided, as the gene expression results are often difficult to interpret.

The timing of the cellular response must also be determined to ensure that the full genomic expression response is observed. Timing of the peak gene expression response can vary widely depending on the experimental conditions: under some conditions the peak response occurs and subsides within minutes of stimulus exposure, and in other situations the majority of the response occurs hours later. The temporal response can be determined by following representative cellular indicators over time, as well as by analyzing genomic expression in a trial time course.

Conducting timecourse experiments with multiple timepoints, rather than single-timepoint microarray studies, offers a number of advantages. Timecourse experiments reveal additional levels of detail, including the choreography of primary and secondary gene expression responses. Timecourses are particularly useful when comparing the responses of different strains to a given stimulus, since the timing of the response may be slightly different in each strain. In addition to these advantages, characterizing a gene's expression over multiple timepoints provides multiple datapoints per gene, and the non-random pattern of gene expression seen for well measured spots increases the confidence in the accuracy of the individual measurements.

Choosing the microarray reference sample. One must also give consideration to the type of reference that will be used on the microarrays, to ensure that enough of this sample is collected during the experiment. In many yeast microarray experiments, mRNA collected from cells growing in logarithmic phase, just before the culture is exposed to the desired stimulus (the so-called "time zero" cells), is used as the reference sample. In other cases a pool of the collected mRNA samples is used as the reference, and even genomic DNA can be used. The required attribute of the reference is that it give significant hybridization signal in all of the microarray spots, so that the relative two-color fluorescence on the micorarrays can be accurately measured. Regardless of the reference used, it is important that the same reference be used on all microarrays in a given timecourse experiment in order to accurately compare the results within the timecourse.

Conducting the experiment

Beginning the experiment. The experiment must be conducted on cells that remain in the same growth phase throughout the experiment, to prevent confounding genomic expression changes. For example, one of the most common problems in yeast genomic expression studies is progression of the cells into the diauxic shift. When the concentration of fermentable sugars in the medium becomes limiting, the metabolic framework of the cell shifts dramatically, leading to alterations in the expression of thousands of genes[1]. The timing of the diauxic shift varies depending on the strain and the exact growth conditions, as cells may consume glucose at rates dependent on their genotype as well as environmental features. Therefore, it is important to investigate the timing of the diauxic shift under each experimental system. This can be accomplished by following the glucose concentration in the medium in a trial experiment to be sure that the glucose concentration does not change appreciably over the course of the experiment.

To start the yeast culture, inoculate fresh medium of appropriate volume from a saturated, overnight culture, and allow the subculture to recover from stationary phase at least two doublings. Begin the experiment at a low cell density, and ensure that the projected cell density at the end of the experiment is well within log phase, to avoid the diauxic shift. As a precautionary measure, the glucose concentration in the culture medium can be measured by saving aliquots of the medium for subsequent analysis (Boehringer Manheim #726251). For long experiments in which the cells may approach the diauxic shift, a chemostat system should be used to maintain the cells in a steady state of growth.

Recording experimental details. Interpreting genomic expression data is often clarified by additional experimental details, however it is sometimes difficult to predict *a priori* which details will be useful. Therefore, it is advisable to record as many experimental details as possible. For example, recording the growth rate and cell viability over the course of the experiment, collecting cell images that may indicate changes in cell morphology and cell-cycle progression, recording relevant nutrient levels in the medium, quantifying drug concentrations when possible, and documenting the timing and method of sample collection can aid in the subsequent analysis of the genomic expression patterns. Any anomalies in the experiment should also be recorded.

Sample collection. Cell culture can be collected by a variety of methods. Cells can be recovered by centrifugation of 50 ml culture samples at 500 rpm for ~3 minutes at room temperature. The cell pellet is immediately resuspended in 3-5 ml Lysis buffer (10 mM Tris-Cl pH 7.4, 10 mM EDTA, 0.5% SDS) in a 50 ml centrifuge tube, flash frozen in liquid nitrogen, and stored at –80°C until RNA preparation. An alternative to the centrifugation method is filtration of the culture through a 1 ?M sterile filter, allowing rapid collection of the cells. Immediately after the filtration, submerge the entire filter with recovered cells in 3-5 ml Lysis buffer, flash freeze, and store at –80°C until RNA isolation.

Collection of the cells should be done in a quick and controlled manner, introducing as few variables into the experiment as possible. Cells should not be washed or unnecessarily handled before collection. Ideally, the temperature of sample collection should be close to the growth temperature; because this is not always possible, it is recommended that cells be collected at room temperature for cultures grown at standard lab temperatures (25° - 30°C). Collection at extreme temperatures, such as on ice, can induce temperature shock in the cells and should be avoided.

RNA isolation and probe generation

The fluorescent probe that will be hybridized to the array is generated by reverse transcription of the RNA isolated from the cell samples and incorporation of fluorescent or modified nucleotides into the nascent cDNA. Either total RNA or poly(A)-selected RNA can be labeled, through a variety of methods described below. Standard RNA handling techniques should be used throughout the RNA isolation procedures, including wearing gloves at all times and using RNase free plasticware. Use of RNA inhibitor-treated plasticware and solutions is optional. Repeated freezing and thawing of the RNA should be avoided, and thawed samples should be maintained on ice to prevent RNA degradation.

RNA isolation. The frozen cell samples are lysed by standard acid-phenol lysis at 65°C: to each frozen cell lysate add one volume of water-saturated phenol (do not allow the lysate to thaw before adding phenol) and incubate at 65°C for 30-45 minutes with periodic vortexing. Centrifuge the emulsion at 10,000 x g for 15 minutes at 4°C to remove the cellular debris; extract the aqueous phase again with water-saturated phenol, then once with chloroform. The total RNA is precipitated from the aqueous phase by adding 0.1 volume 3 M Sodium acetate and 2.5 volumes

Ethanol, and incubated >1 hour at –20°C. After centrifugation at 20,000 x g for 30 minutes at 4°C, the washed and dried RNA pellet should be resuspended in ~0.5 to 1 ml TE buffer. The final concentration, estimated by measuring the absorbance at 260 nm, should be 1-2 mg/ml, and the ratio of absorbance at 260 nm *versus* 280 nm should be 2.0-2.3 for protein-pure RNA. The RNA can be aliquoted and stored at -80°C.

For isolation of poly-adenylated RNA, various kits are available. The mRNA can also be recovered using oligo-dT cellulose (Ambion #10020)[36]. Prepare the cellulose by washing 1 g of cellulose 2-3 times in 2X NETS buffer (200 mM NaCl, 20 mM Tris-Cl pH 7.4, 20 mM EDTA, 0.4% SDS), and resuspend in 10 ml 2X NETS buffer. To bind the sample to the resin, dilute 1 mg of total RNA into 1 ml of TE, and combine with 1 ml of the suspended oligo-dT cellulose in a 2 ml disposable column (Biorad #732-6008). Allow the column to rotate on a small rotor for 1 hour at room temperature to allow the poly-adenylated RNA to bind the resin. To begin column washes, break the bottom of each column to allow liquid to flow through, and mount the columns onto a 15 ml Falcon tube. Wash each column 7 times with 1 ml 1X NETS buffer. Transfer the column to a fresh Falcon tube for elution, then elute 2 times with 700 μl 1X ETS buffer (10 mM Tris-Cl pH 7.4, 10 mM EDTA, 0.2% SDS) *heated to 70°C.* To remove any granules of cellulose that escape into the eluate, extract the eluate once with one volume chloroform, then precipitate the RNA by adding 0.1 volume 3M Sodium acetate and 1 volume Isopropanol, and incubate overnight at –20°C. Collect the RNA by centrifugation and resuspend the washed pellet in 20-30 μl TE to yield a concentration of ~1 μg/μl, quantifiable by measuring the absorbance at 260 nm. The purity of the mRNA can be assessed by electrophoresis of an equal mass of total RNA and the purified poly-adenylated RNA; although the two prominent bands of ribosomal RNA will likely remain in the poly-adenylated sample, an enrichment of poly-adenylated RNA is evident by a smear ranging from ~500 basepairs up the gel. Significant intensity at the bottom of the gel (< 300 basepairs) indicates RNA degradation; the sample should not be used for microarray probe generation.

In some cases it may be necessary to amplify the mass of mRNA to generate enough template for probe preparation. Amplification from total RNA or poly(A)-isolated RNA involves generating a double-stranded cDNA that is subsequently transcribed from a linker sequence that has been incorporated into the cDNA, using T7 polymerase. Complete details on the amplification protocol can be found elsewhere[37,32,33].

Microarray probe generation.

Fluorescent cDNA probe can be generated through a variety of methods. The probe can be generated from mRNA, total RNA, or DNA by incorporating fluorescent or modified nucleotides into the cDNA product. The protocols for generating probe using fluorescent nucleotides (Cy-dUTP labeling) or modified nucleotides (amino-allyl labeling) are listed below. Regardless of the protocol, consistency in the labeling should be maintained. When labeling many samples to be compared, it is advisable to create a cocktail of reaction mix and perform the labeling reactions at the same time.

Reverse transcription using Cy-dUTP. Two fluorescently-labeled probes are generated for each microarray experiment, incorporating one of two modified dUTP nucleotides (Cy3-dUTP or Cy5-dUTP) through reverse transcription using StrataScript reverse transcriptase (Stratagene #600065). Combine either 15 ?g of total RNA or 2 ?g mRNA with 5 ?g of an “anchored” oligo dT primer of the sequence 5’ (T)$_{15}$-V-N 3’, where V is any nucleotide but T and N is any nucleotide. The two additional nucleotides on the 3’ end of the oligo dT primer promote priming at the junction between the transcript and the poly(A) tail. Adjust the volume with TE buffer to 15.5 ?l. Denature the RNA mixture by incubating at 70°C for 10 minutes then immediately place on ice. In the meantime, a reaction cocktail is prepared and stored on ice: for each reaction, add to the cocktail 3 ?l 10X StrataScript buffer, 3 ?l 100 mM DTT, 0.5 ?l 50X dNTP mixture (10 mM dTTP, 25 mM dATP, dCTP, dGTP), 2 ?l ddH$_2$O, and 3 ?l StrataScript reverse transcriptase. To each denatured RNA mixture, add 3 ?l 1 mM Cy3-dUTP or Cy5-dUTP

(Amersham) and 11.5 ?l of the reaction cocktail for a final reaction volume of 30 ?l. Incubate the reaction at 42°C for 2 hours. After the reaction has completed, the unincorporated fluorescent nucleotides must be removed from the reaction. Dilute the reaction mixture with 500 ?l TE buffer, and add to a Microcon 30 spin filter (Amicon #42410). Spin in a microfuge approximately 9 minutes until the volume is reduced to < 5 ?l. Collect the concentrated probe by inverting the filter into a fresh tube, then adjust the final concentration of the probe to 10 ?l with TE buffer. (Unincorporated Cy-dUTP can be collected from the flow through for reuse, as described elsewhere [32].) The probe can be stored at this stage for a few days at 4°C but should not be frozen.

The labeling reaction can also be primed with random primers, as opposed to or in combination with the oligo dT primer that specifically binds to the poly(A) RNA tail. The protocol is nearly identical to the protocol described for using oligo dT primer, except that 5 ?g of random octamers is used (either alone or in addition to 5 ?g of the anchored oligo-dT primer) and that the mixed reaction must be incubated for 10 minutes at room temperature before transferring to 42°C.

In addition to labeling RNA samples, DNA can also be used to generate fluorescent probes using Klenow polymerase[38]. Adjust the volume of a 2 ?g DNA sample to 21 ?l with TE buffer. Add 20 ?l of 2.5X reaction cocktail (125 mM Tris-Cl pH 6.8, 12.5 mM Magnesium chloride, 25 mM 2-Mercapthoethanol, 0.75 ?g/?l random octamer primers). Incubate the solution at 94°C for 5 minutes to denature the probe, then chill on ice. Add 5 ?l of a 10X dNTP mixture (0.6 mM dTTP, 1.2 mM dATP, dCTP, dGTP), 3?l of 1 mM Cy3-dUTP or Cy5-dUTP (Amersham), and 1 ?l (20 – 50 units/?L) Klenow DNA polymerase for a final reaction volume of 50 ?l. Incubate the reaction for 2 hours at 37°C, then quench the reaction by adding 5 ?l 0.5 M EDTA. As described above, purify the probe by dilution and filtration in a Microcon 30 filter.

Reverse transcription using amino-allyl-modified nucleotides. An alternative and more economical method of generating fluorescent probes is to chemically couple the fluorescent dye to the cDNA after reverse transcription. By incorporating amino-allyl-modified nucleotides into the nascent cDNA, activated dyes can be subsequently coupled to the amino moieties on the cDNA. A variety of fluorescent dyes can be attached to the amino moieties, including NHS ester Cy3 and Cy5 (Amersham #23001 and #25001). The NHS ester Cy dyes should be suspended in 16 ?l fresh DMSO, divided into 1 ?l aliquots (providing dye for one labeling reaction), and dried completely in a speed vacuum. Because the dyes are labile in solution, it is important to be sure the samples are completely dry. The dried dyes can be stored under desiccation at 4°C. Alternatively, each tube of dye can be suspended in 8 – 12 ?l DMSO and used immediately for coupling, using 1 ?l dye per reaction.

The amino-allyl labeling method consists of three steps: cDNA synthesis, coupling of fluorescent dyes to the cDNA, and quenching of the reaction to prevent cross coupling of the dyes once the samples are combined[39]. For the reverse transcription, combine 2 ?g mRNA or 15 ?g total RNA with 5 ?g anchored oligo-dT primer in a volume of 15.5 ?l, denature the RNA at 70°C for 10 minutes, and place the sample on ice. To the denatured RNA solution add 14.5 ?l of the reaction cocktail: for each reaction, add to the cocktail 3 ?l 10X StrataScript buffer, 3 ?l 100 mM DTT, 0.5 ?l 50X dNTP mixture (10 mM 5-(3-Aminoallyl)-2'-deoxyuridine 5'-triphosphate (Sigma #A0410), 15 mM dTTP, 25 mM dATP, dCTP, dGTP), 5 ?l ddH$_2$O, and 3 ?l StrataScript reverse transcriptase (Stratagene #600065). The final reaction volume should be 30 ?l. Allow the reaction to proceed at 42°C for 2 hours. After completion of the cDNA synthesis, the remaining RNA can be hydrolyzed by adding 10 ?l 1 N Sodium hydroxide and 10 ?l 0.5 M EDTA. After a 15 minute incubation at 65°C, the solution in neutralized by adding 25 ?l 1M HEPES pH 7.5 or 10 ?l 1 N Hydrochloric acid. At this point the samples can be stored at $–20^{o}$C for months.

Before the chemical coupling step, all Tris must be removed from the reaction. Dilute the neutralized reaction mixture into 500 ?l ddH$_2$O in a Microcon 30 spin filter (Amicon #42410) and centrifuge for 8 – 10 minutes until the volume is <10 ?l. The filtration is repeated 2 more times, each time by adding 500 ?l ddH$_2$O to the filter and repeating the centrifugation. After recovering the concentrated cDNA into a fresh tube, dry the sample in a speed vacuum or continue the microcon filtration until the volume is ~4 ?l. The dried or liquid samples can be stored at -20°C.

To couple the fluorescent dyes to the modified cDNA, resuspend the dried cDNA in 9 ?l 0.05 M Sodium bicarbonate buffer pH 9.0, or add 4.5 ?l of 0.1 M Sodium bicarbonate buffer to 4.5 ?l of the concentrated sample for a final concentration of 0.05 M Sodium bicarbonate (verify the pH of the buffer before each use), and incubate at room temperature for 10 minutes to resuspend. After mixing well, transfer this solution to the dried aliquot of activated Cy3 or Cy5 dye or to 1 ?l of the freshly-suspended dye and mix well. Allow the reaction to proceed for 1 hour at room temperature in the dark.

Finally, the reaction must be quenched to prevent cross coupling of the Cy dyes when the labeled samples are combined. To each reaction, add 4.5 ?l 4 M Hydroxylamine and incubate for 15 minutes at room temperature in the dark. After quenching the reaction, combine the Cy3- and Cy5-labeled samples and purify the mixture using a QIAquick PCR purification kit (Qiagen #28104), as described in the kit protocol. The eluate should be concentrated by microcon filtration or drying in a speed vacuum, and the sample should be resuspended in ddH$_2$O for a final volume of 10 ?l and prepared for hybridization as described below. Faint color in the concentrated sample indicates that the labeling reaction was successful. When color is not evident, the fluorescence can be checked by spotting <0.5 ?l of the probe onto a glass microscope slide and scanning the spot to verify fluorescence.

Microarray hybridization and data acquisition

Microarray hybridization. To prepare the probe for hybridization to a ~22x22 mm microarray, combine 5 ?l of each of the Cy-dUTP labeled samples or 10 ?l of the combined amino-allyl labeled sample, 1 ?l of 10 mg/ml polyA carrier (Sigma #P9403), 2.3 ?l 20X SSC, and 0.36 ?l 10% SDS for a final volume of 14 ?l (if the microarray is larger than 22x22 mm add to the 10 ?l of probe solution 2.6 ?l polyA, 5.0 20X SSC, and 0.78 ?l 10% SSD and adjust the final volume to 30 ?l with water). Denature the probe by heating at 94°C for 3 minutes, then cool to room temperature by rapidly transferring to a microfuge and spinning for 10 seconds (avoid adding bubbles during handling). Do not chill the probe on ice as salt will precipitate from the solution.

Prepare the hybridization chamber (Corning #2551): place the microarray into the chamber and add ~20 ?l 3X SSC to each of the hybridization wells in the chamber and an additional ~30 ?l of 3X SSC divided into isolated drops on the edges of the microarray. Insufficient volume will result in inadequate chamber hydration during hybridization, causing the probe to dry out under the coverslip. Select multiple microscope slide coverslips of the appropriate size (Corning #2865) that appear free of dust, chips, and scratches, and prop them for easy access with a tweezers.

Adding the probe to the microarray and sealing with the coverslip requires some experience and personal technique. It is advisable to practice the following procedure before hand. To the center of the microarray, deposit 12-15 ?l of the denatured and cooled probe for 22x22 mm arrays, or 28-30 ?l probe for 22x44 mm microarrays. Bubbles can prevent proper hybridization, and therefore they should be avoided and removed when possible. Large bubbles can be popped with the corner of a coverslip, but great care should be taken not to touch the microarray surface. Quickly place the coverslip directly over the deposited probe and gently drop or place the coverslip over the array. The coverslip should not be moved so as to prevent scratching of the microarray surface, however in unavoidable cases it can be gently positioned by sliding. Close the hybridization chamber and gently and quickly submerge the chamber in a 65°C water bath. For optimal signal, hybridize the arrays for 7 - 16 hours. For consistent results, the hybridization conditions, including hybridization time, should be maintained for all microarrays.

Microarray washing. After the hybridization is complete, the unbound fluorescent probe and detergent are removed from the microarrays in a series of three washes of increasing stringency. Prepare three wash solutions in glass slide chambers: for the first wash, dilute 18 ml 20X SSC and 1 ml 10% SDS with ddH$_2$O to a final volume of 350 ml; the second wash is identical to the first wash only without any SDS; for the final wash add 1 ml 20X SSC to 350 ml ddH_2O. After removing the arrays from the water bath, disassemble the hybridization chamber and quickly set the array into a metal slide rack submerged in the first wash. Gently and slowly move the slide rack under the wash solution until the coverslip slides off, taking care not to scratch the microarray surface with the coverslip. When the coverslip has separated, removed each microarray individually from the first wash, allow the solution to drain from the slide, and place the slide into a separate slide rack submerged in the second wash solution. Residual SDS can increase the background fluorescence on the arrays, so minimizing carryover of the first wash is recommended. When disassembling multiple hybridizations, the microarrays can remain in the second wash until all chambers have been disassembled. Plunge the rack in the second wash for a few seconds, then remove the entire slide rack from the second wash and transfer to the final, low stringency wash. Incubate the slides in the final wash for 5 minutes with slight agitation on a platform shaker, then dry the slides by centrifugation at 500 rpm for 3 minutes in a tabletop centrifuge before scanning. The arrays can be stored overnight in the dark at room temperature before scanning, however longer storage may result in diminished Cy5 signal on the arrays.

Data acquisition. Retrieving the microarray data involves a “scanning” process, in which the fluorescent dyes hybridized to the array are excited with a laser and the emitted fluorescence for each of the dyes is recorded. Among the most popular of the scanning laser devices available for purchase is the GenePix scanner from Axon Instruments. Dual lasers simultaneously excite the Cy3 and Cy5 dyes at 532 nm and 635 nm, respectively, and the emitted fluorescence for each of the dyes is measured independently (different dye-specific laser and filter sets are also available). The parameters of the scanner can be adjusted during scanning, as described in the GenePix manual. The microarrays should be scanned at settings that maximize the intensity of the spot fluorescence signal within the range of signal detection, rather than settings that result in normalized signal between the two channels.

Normalization of the signal intensities should be done mathematically after scanning and data extraction, as described below.

Once the microarray has been scanned, the data are extracted through a procedure known as “gridding”. The GenePix software that accompanies the scanner can be used to place a grid of circles over the microarray fluorescence image, such that each spot is encircled. The fluorescence signal corresponding to each dye is then quantified: the local background signal measured just outside of each grid circle is subtracted from the signal measured within the circle, providing background-subtracted values for each DNA spot. Flawed spots, including those surrounded by abnormally high background signal, spots obscured by dust, or areas of bled and contaminated spots, can be removed from the dataset by manually “flagging” bad spots. Because spots with weak fluorescent signal are difficult to quantitate, they are automatically flagged by the GenePix gridding software.

A critical aspect of extracting microarray data is correct identification of each microarray spot. Identifying the spots with a unique ORF or sequence name requires deconvolution of the list of DNAs in the 384-well plates, generated from the original 96-well plates of PCR products or 70mer oligos, that were printed onto the microarrays. This deconvolution process involves two steps: converting the list of DNAs in 96-well format into a 384-well format, and then converting the list of DNAs in the 384-well plates into the list of ordered spots on the microarrays. Conversion of the DNA sample order in the 96-well format to the 384-well configuration is dependent on the configuration used to transfer four 96-well plates into each 384-well plate, while deconvoluting the order of DNAs in 384-well format to the microarray spot order is dependent on the number of tips used in the printing and the order that the plates were spotted onto the arrays. Both of these deconvolutions can be performed using the GenePix software. In both cases, separate lists of DNA identifications are created for each 96-well or 384-well plate to identify the DNA in the consecutive plate wells. The required parameters (including plate transfer configuration or microarray printing details) are set and the separate plate lists are selected in the appropriate order. The resulting spot list can be associated with the microarray grid before data extraction as described in the GenePix manual. A note of caution is that it is *imperative* that the microarray spot identification is correct. When creating a new spot list for each set of microarrays, careful verification of the list should be done, ideally by hybridizing an identical sample to a newly printed array and a previously verified microarray to be sure that the data agree. Correct spot identification can also be checked by verifying the expected expression ratios for internal control spots in the experiment, including auxotrophic markers, gene deletions, or gene overexpression present in one of the labeled samples hybridized to the microarrays.

Before recording the relative background-corrected fluorescence within each spot, the fluorescence intensity corresponding to the two dyes must be normalized. Because the two fluorescent dyes are scanned and detected independently, the overall signal in the two channels must be normalized to each other. One method of normalization is based on the assumption that most genes will not change in expression in that experiment. The fluorescence signal can be normalized, based on this assumption, by adjusting the overall fluorescence intensity in one of the channels by a normalization factor, such that the relative signal intensity in most of the microarray spots is equal. Another method of signal normalization is to dope the Cy3 and Cy5 labeling reactions with known quantities of an empirically determined set of control DNA fragments that are also present on the microarray. The fluorescence signal in the channels can then be adjusted to achieve the known ratios for those control microarray spots.

Databases. Microarray experiments quickly generate massive datasets that require efficient means to store, explore, and retrieve the data. Efficient databases allow the storage of each set of microarray files (including the scan images, grid file, and extracted data file), details regarding the microarray used (printing details including print batch, identification of the microarray spots, etc.), and specific details of the experiment (strain genotype, medium, culture conditions, and specific experimental details). A publicly available database can be downloaded at http://www.microarrays.org/software.html [40,33].

Data analysis and interpretation. The existing challenge is extracting biological insights from the massive genomic expression datasets generated by microarray studies. In the subsequent chapter by Eisen, a variety of computational methods used in the analysis of genomic expression data are described. Effective interpretation of the data requires not only computational techniques, but also a constant awareness of the experimental conditions. Before formalizing hypotheses about the cellular signals that provoke gene expression changes, the experimenter should consider the primary *versus* secondary cellular responses to the experimental conditions, possible pleiotropic effects of the cells' environment, and the specificity of the observed gene expression responses to the experimental conditions.

In many cases, interpreting the genomic expression program triggered by one set of experimental conditions is clarified by comparing the results to other conditions. For example, to identify cell cycle-regulated transcripts, Spellman *et al.* analyzed genomic expression data recovered from four different experiments, in which cells were synchronized by different means, to identify the cell-cycle regulated genes common to all four experiments while filtering out genes whose expression was specifically affected by the mechanism of synchronization [3]. In another study by Gasch *et al.* that followed the genomic expression responses of cells to different environmental changes, it became apparent only when the data from the different experiments were compared that much of each of the genomic expression responses was not specific to the individual conditions, but rather was part of a general stress response that was triggered by all of the experiments [8].

Troubleshooting microarrays

Measuring the relative spot fluorescence on microarrays with bright spots of high signal-to-noise is quantitative and highly reproducible, however a variety of common flaws reduce the reliability of quantitation. The most frequently encountered microarray blemishes are summarized below, with suggested techniques for improving microarray quality.

Weak spot signal. Weak global fluorescence signal most commonly arises due to inefficient labeling of the sample. RNA of insufficient quality or quantity prevents proper probe generation, and a first step toward identifying weak signal problems is to check the quality of the RNA sample. Probe fluorescence can be increased simply by increasing the amount of sample labeled in the reaction; when the sample is limiting, methods such as amplification may be required to generate enough template for the reaction. Alternate labeling protocols may also increase the probe intensity. For example, mRNA labeled with random primers or a combination of random primers and the specific oligo dT primer results in brighter probe than using the oligo dT primer alone. Recent developments with alternate labeling systems also increase the signal intensity by increasing the number fluorescent dye molecules incorporated into the probe (Genisphere #A100736 and #A100746).

Other microarray flaws result in weak local signal. Circular areas of weak signal may reflect the presence of bubbles during the hyrbridization. Areas of weak signal near the perimeter of the microarray, outlined by extremely bright fluorescence intensity, arise when the probe dries out during hybridization due to insufficient chamber hydration. This issue can be eliminated by increasing the volume of 3X SSC added to the microarray chamber during hybridization. Streaks of low signal across the microarray result from scratches, most often introduced during removal of the microarray coverslip.

High background signal. Just as spots with weak signal are more difficult to quantitate, spots surrounded by high background signal are equally susceptible to measurement error. High global background can result from bad post-processing of the microarrays before hybridization, since fluorescent probe DNA may bind to the microarray surface if the unbound poly-lysine groups are not sufficiently blocked. Another suspect when global background is high is the probe mixture: inappropriate concentrations of the probe components, particularly SDS, can increase the global background signal on the microarrays.

High local background can result in a variety of impressive but undesirable patterns on the arrays. Residual SDS, introduced during washings of the hybridized microarrays, is detected as swirly patterns of green fluorescence. Residual SDS can sometimes be removed after scanning by further washing the arrays in a warm solution of the final wash. More problematic are the bright, multicolored background patterns that arise due to bad poly-lysine coated glass. These patterns, often accompanied by areas of very high background fluorescence and no spot fluorescence, may reflect poly-lysine-coated glass that is over aged. It is strongly advised that test hybridizations be performed on representative poly-lysine slides from each batch before investing the effort to print complete microarrays.

Comet tails, speckles, and other blemishes. Another problem caused by bad poly-lysine slides or ineffective post-processing is comet tails trailing off of the DNA spots. These fluorecent tails likely result when DNA from the microarray spots migrates across the slide. Although the exact cause of this blemish is unclear, its source may be related to the chemical post-processing step. The chemical blocking step should be initiated rapidly, and the rack of slides should be plunged into the blocking solution, rather than pouring the solution over the dry slides. The comet tail phenomenon may be exacerbated when the poly-lysine coated glass is insufficiently aged, resulting in poor hydrophobicity of the surface. Poor hydrophobicity also promotes the bleeding of spots on the microarray during printing. Bleeding between spots can be minimized by reducing the rehydration time in the post-processing procedure, and by increasing the salt concentration in the hybridization chamber from 1X to 3X SSC.

Speckles of high fluorescence on the microarrays can reduce the accuracy of measurements and also interfere with automatic gridding software. Dust on the microarrays is most often the culprit. Although dust particles may be fluorescent themselves, fluorecent probe can also bind to the particles. Care should be taken when handling the microarrays, and they should be stored in a dust-free environment. Be sure all equipment is free of dust, including glassware, centrifuges, and the microarrayer itself.

Another source of speckled arrays is precipitation of the probe components before or during hybridization. Precipitation of salt and SDS can occur, especially if the probe is chilled (or is allowed to freeze during speed vacuuming). Therefore, the probe should not be stored once the salt and detergent have been added, and the solution should not be chilled below room temperature after denaturation.

Summary

The exploration and characterization of yeast genomic expression programs is providing a wealth of information about yeast biology, as well as other organisms. The intriguing biology of yeast species invites characterization of genomic expression patterns to illuminate the details of cellular physiology. But in addition to its value as an interesting organism, yeast maintains its role as an excellent model in which to characterize genomic expression programs. Microarray studies are quickly spreading to plant, animal, and microbial organisms that remain in the early stages of characterization. The extensive knowledge of yeast biology, as well as the relative ease in which yeast studies can be performed and controlled, facilitates interpretation of the genomic expression data. Importantly, existing information about yeast biology, including functional annotations for each gene, is captured and efficiently presented in databases such as the *Saccharomyces* Genome Database (SGD)[41], the Munich Information Center Yeast Genome Database (MIPS)[42], the Yeast and Pombe Protein Databases (YPD and PPD, respectively)[43,44], and others. A number of databases also allow the exploration of published genomic expression studies, including the “Expression Connection” at SGD and the Microarray Global Viewer (yMGV) organized by Marc *et al.*[45]. Consulting these databases to retrieve known details about gene function and regulation vastly facilitates interpretation of the genomic expression data, allowing biological hypotheses to be formulated and tested. These hypotheses can be applied to other organisms that may execute genomic expression programs similar to those seen in yeast. Furthermore, as more genomic expression studies in multiple organisms emerge, large-scale data comparisons can be conducted, within and across organisms. Incorporating the results of yeast studies into such comparisons is certain to increase our understanding about the function, regulation, and evolution of genomic expression programs.

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